Intracellular Signaling Pathways in Rheumatoid Arthritis

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Abstract
Dysfunctional intracellular signaling involving deregulated activation of the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) and “cross-talk” between JAK/STAT and the stress-activated protein kinase/mitogen-activated protein kinase (SAPK/MAPK) and Phosphatidylinositol-3-kinase/AKT/mammalian Target of Rapamycin (PI-3K/AKT/mTOR) pathways play a critical role in rheumatoid arthritis. This is exemplified by immune-mediated chronic inflammation, up-regulated matrix metalloproteinase gene expression, induction of articular chondrocyte apoptosis and “apoptosis-resistance” in rheumatoid synovial tissue. An important consideration in the development of novel therapeutics for rheumatoid arthritis will be the extent to which inhibiting these signal transduction pathways will sufficiently suppress immune cell-mediated inflammation to produce a lasting clinical remission and halt the progression of rheumatoid arthritis pathology. In that regard, the majority of the evidence accumulated over the past decade indicated that merely suppressing pro-inflammatory cytokine-mediated JAK/STAT, SAPK/MAPK or PI-3K/AKT/mTOR activation in RA patients may be necessary but not sufficient to result in clinical improvement. Thus, targeting aberrant enzyme activities of spleen tyrosine kinase, sphingosine kinases-1, -2, transforming growth factor β-activated kinase-1, bone marrow kinase, and nuclear factor-xB-inducing kinase for intervention may also have to be considered.

Keywords: Apoptosis; Arthritis; Cartilage; Cell survival; Inflammation; Protein kinase; Signal transduction; Small molecule inhibitor; Synovium

Introduction
The concept was so elegant in its simplicity that it was surprising that directly targeting intracellular signaling pathways in arthritis to restore normalcy to dysfunctional immune-mediated inflammation took so long to have finally reached the clinic. Now that the novel small molecule Janus Kinase-1, -3 (JAK1/3) inhibitor, tofacitinib, which was recently approved by the US Food & Drug Administration for the treatment of moderate-to-severe rheumatoid arthritis (RA) [1] is available for treating RA patients in the general population, it is likely that this monumental achievement in the development of an inhibitor that targets a specific class of protein kinases will further revolutionize the medical therapy of RA.

Drugs Employed in the Medical Therapy of RA
Presently tofacitinib is essentially restricted for use in RA patients who are unresponsive or have become refractory to therapy with conventional disease-modifying anti-rheumatic drugs (DMARDs) or to treatment with anti-rheumatic biological agents. The conventional DMARDs included in the pharmaceutical armamentarium for RA are corticosteroids, salicylates and non-steroidal anti-inflammatory drugs [2], anti-malarial drugs [3] methotrexate [2], and leflunomide [4]. Depending on whether or not RA patients demonstrate a clinically significant response to conventional DMARDs, RA patients may then also be treated with anti-rheumatic biological drugs. Included in this group are tumor necrosis factor (TNF) inhibitor monotherapy [5,6], TNF inhibitor in combination with methotrexate [4] or leflunomide [7], anti-IL-6-receptor binding inhibitor [8-11] or anti-IL-6 receptor binding inhibitor in combination with methotrexate [10,11], anti-IL-1 inhibitor [12,13], anti-T-cell [14,15] or anti-B-cell [16,17] biologicals. RA patients treated with tofacitinib plus methotrexate has also recently been reported [18].

The “Standard of Care” for RA Indicates that the Use of Multiple Biological Drugs is Prohibited
At the present time, employing multiple biological drugs in a single RA patient is prohibited [19]. In addition, serious concerns continue to exist regarding long-term monotherapy with these biological drugs. RA patients are already known to be at higher risk for co-morbid conditions associated with their disease. Moreover, developing these co-morbitities appears to be independent of the defined risk factors for these conditions in the general population. Thus, long-term therapy with biological drugs may increase the relative risk to RA patients for developing serious infections [20-22], malignancies [20,23] as well as a worsening of cardiovascular disease, especially in those RA patients with pre-existing congestive heart failure [24], the latter condition wherein therapy with biological drugs is contraindicated [25].

Future Drug Therapies for RA
An important concern in developing novel RA therapeutics going forward will be the extent to which inhibition of the JAK/Signal Transducers and Activators of Transcription (STAT) pathway alone will sufficiently suppress immune cell-mediated inflammation to produce a lasting clinical remission. In that regard, the majority of the evidence now having been accumulated over the past decade indicated that merely suppressing pro-inflammatory cytokine-mediated JAK/STAT activation in RA patients may be necessary but not sufficient to result in clinical remission.

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Received June 12, 2013; Accepted August 13, 2013; Published August 19, 2013


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“Personalized Molecular Medicine”

Consistent with this view, we predict that the most efficient way for future RA drug development will be to focus on producing orally-administered SMIs with high selectivity and specificity for individual protein kinases. This paradigm could prove even more fruitful if a “personalized molecular medicine” approach was developed whereby the repertoire of elevated pro-inflammatory cytokines in both the serum and synovial fluid of individual RA patients was completely defined prior to beginning drug therapy and a specific protein kinase SMI chosen on the basis of which cytokine and/or signaling pathway repertoire was involved.

In light of this contention, we recently suggested that dysfunctional intracellular signaling involving protein kinase pathways other than JAK/STAT as well as “cross-talk” between JAK/STAT and the stress-activated protein kinase/mitogen-activated protein kinase (SAPK/MAPK) and Phosphatidylinositol-3-kinase/AKT/mammalian Target of Rapamycin (PI-3K/AKT/mTOR) pathways is likely to be completely dampen immune-mediated inflammation and cartilage destruction in RA [26-28]. Supporting this viewpoint are the results of an analysis involving the therapy of active RA with the anti-IL-6 receptor inhibitor, tocilizumab [29]. Thus, in a random 90% sample of data from the ‘Tocilizumab Safety and the Prevention of Structural Joint Damage Study’ (LITHE) clinical trial of patients with active RA treated with methotrexate, the results revealed a surprisingly clear-cut dissociation between suppression of inflammation induced by tocilizumab as measured by the ‘The Genant-modified Sharp Score’ (‘TGSS’), simplified disease activity score index, swollen joint count and C-reactive protein levels which were all reduced from baseline in the tocilizumab-treated group, and evidence of synovial joint cartilage destruction, as measured by changes in joint space narrowing by X-ray analysis. These results also showed that RA patients achieving clinical remission by both objective and subjective criteria in the placebo or tocilizumab arm who had initial low disease scores, low TGSS, minimal bone erosion and near-normal joint space narrowing benefited more from treatment with tocilizumab or placebo than RA patients with higher initial disease activity scores in either the placebo or tocilizumab arm of this clinical trial. Therefore, when all of the data from clinical trials such as this one are combined with previously reported basic and clinical research data [26,27,30], the results suggest that multiple drug treatment strategies may have to be considered in order to suppress the progression of articular cartilage damage in RA, which appears to result from a mechanism quite distinct from the one that regulates changes in biomarkers of inflammation, bone erosion and cell survival.

Intracellular Signaling Regulates Immune-cell, Synoviocyte Survival, and Cartilage Apoptosis in RA

In addition to these above considerations, there is now mounting evidence that several protein kinases are deregulated in RA as well as in autoimmune disorders in general. Thus, dysfunctional intracellular signaling induced by pro-inflammatory cytokines was shown to be responsible for aberrant immune-cell survival [31-33], articular chondrocyte apoptosis [33,34] and/or “apoptosis-resistance” of cells in RA synovial tissue [35-38]. Abnormalities in the aforementioned JAK/STAT, SAPK/MAPK and PI-3K/AKT/mTOR pathways [26,27,39-41] as well as aberrant activities in spleen tyrosine kinase (Syk) [42,46], the sphingosine kinases, SphK1 and SphK2 [47-52], transforming growth factor β-activated kinase-1 (TAK1) [53], bone marrow kinase (BMX) [54] and nuclear factor-κB-inducing kinase (NIK) [55] have all been found in patients with active RA. To illustrate this point, Gottar-Guillier et al. [54] showed that BMX activity was a requirement for p38 kinase and JNK phosphorylation as well as for the activation of NF-κB [55]. Therefore BMX may be responsible for regulating the activation of p38, JNK and NF-κB, all of which are critical to the inflammatory response cell survival and apoptosis.

Thus, over-expression of protein kinase genes or changes in protein kinase activity would result in, or promote exuberant cell survival and/or resistance to apoptosis, or both. Of note, SMIs for a few of these protein kinases are already in development for the therapy of RA [51,56,57].

Which Pro-Inflammatory Cytokines are Elevated in the Sera and Synovial Fluids of RA Patients?

There is now persuasive evidence that the level of a particular pro-inflammatory cytokine in sera and synovial fluid reflects their role in the pathogenesis and progression of RA. Thus, TNF-α and several of the interleukins (IL), including, IL-1, -6, -7, -8, -12/23, -15, -17, -18, -32, -35 and proteins of the interferon (INF) family were found to be elevated in RA sera [58-64] with a matching elevated repertoire of these cytokines in RA synovial fluid [28,65-69]. Of note, the cytokines produced by the T₅₂ subset, identified by the expression of the GATA₃ transcription factor, which drives production of the anti-inflammatory cytokines, IL-4, -5 and -13, were found at reduced levels in RA [70]. Therefore, based on these results it is tempting to design experimental strategies to rigorously define which of several possible intracellular signaling pathways are activated or suppressed by the biological activities of these elevated levels of pro-inflammatory cytokines. Likewise we can envision developing experimental systems to test the hypothesis that production of anti-inflammatory cytokines can be potentially increased by targeting activation of signaling cascades responsible for the expression of IL-4, -5, -13 and IL-10 genes.

Activation of the JAK/STAT Pathway

Activation of STAT proteins occurs after the interaction of pro-inflammatory, anti-inflammatory cytokines or growth factors with specific membrane-bound receptors on cells which results in JAK phosphorylation [71]. In this regard, STAT-responsive gene transcription is pertinent to perpetuating inflammation and progression of RA joint destruction [71]. To summarize, the principal effects of JAK/STAT pathway activation by those pro-inflammatory cytokines which are present in elevated amounts in RA sera and synovial fluid, and including, IL-2, IL-3, and IL-19/IL-20, is to increase the gene expression of IL-2R, -3, -4, -6, gp130 (a component of the IL-6 trans-signaling complex), IL-10, -18R1, INF-γ, Oncostatin M (OSM) and TNF-α. Recent analyses have shown that IL-17A [72-74], IL-19 [75,76], and IL-20 also play an influential role in the context of JAK/STAT pathway activation [72,76] and in the progression of RA pathology which make these findings particularly noteworthy. In particular, Moran et al. [74] showed that IL-17A increased matrix metalloproteinase (MMP)-1, -2, -9, and -13 by RA synovial tissue explants, RA synovial fibroblast cultures, articular cartilage and cultured chondrocytes. This MMP repertoire was shown to be significant in driving the degradation of articular cartilage extracellular matrix (ECM) proteins in RA [77]. Moreover, together with OSM and TNF-α, treatment with IL-17A resulted in a skewing of the ratio of MMPs to tissue inhibitor of matrix metalloproteinases (TIMPs) favoring MMPs. However, cartilage explant ECM protein was only partially degraded in the presence of IL-17A, but when OSM and TNF-α was added, cartilage ECM protein degradation was complete. Interestingly, only 28% of those RA patients in this patient cohort had...
SAPK/MAPK Pathway Activation

From a functional perspective the SAPK/MAPK signaling pathway is composed of the 5 p38 kinase isoforms, p38α, p38β, p38γ, and p38δ, extracellular signal-regulated protein kinases-1, -2, -3, -4, -7 (ERK-1, -2, -3, -4, -7) and C-Jun-N-terminal kinases, JNK1, JNK2 and JNK3 [78]. Complete activation of SAPK/MAPK signaling generally is dependent on the activity of upstream kinases, namely, MEK, MKK4 and MKKK7 [79], although there is some evidence that upstream kinase kinase-independent SAPK/MAPK activation can also occur [80].

A renewed focus on the role of the SAPK/MAPK signaling cascade in RA is driven by its significant role in regulating the cellular responsiveness to various pro-inflammatory cytokines including, MMP gene expression [40,81], NF-κB activation [82], and cell survival and apoptosis [79,83]. However, inhibition of p38α using an orally-administered SMI although showing promise in preclinical studies in animal models of RA [84] then failed to show efficacy in adult RA clinical trials [85]. This result may shift the focus of SMI research and development towards ERK 1/2 and JNK or to other components of SAPK/MAPK signaling implicated in the survival or apoptosis of immune-cells and activated synoviocytes.

SAPK/MAPK Activation: Effects on Inhibitor of Apoptosis Proteins, IKKβ, c-Myc and TNFR

Studies on the inhibitor of apoptosis proteins (IAP) have shown that they are critical regulators of immune-cell death in RA [31] as well as inflammation in general [25]. With respect to defects in the innate immune system in RA, the activity of several pattern recognition receptors (PRRs) including, Toll-like receptor-4 (TLR4), nucleotide-binding oligomerization domain 1, (NOD1), nucleotide-binding oligomerization domain 2 (NOD2) receptors and retinoic acid-inducible gene (RIG-1) receptor were shown to be IAP-dependent [86]. Importantly, recent evidence showed that cellular-IAP1 (cIAP1), cIAP2 and X-chromosome-linked IAP (XIAP) (Figure 1), contained ubiquitin ligase activity [87,88] and by this mechanism regulate p38, JNK and NF-κB [89] activity and vice versa [90].

Interestingly, c-IAPs are required to recruit IKKβ (Figure 1) the activation of TNFR complexes (Figure 1). In summary, TNFR complexes regulate the activation of the non-canonical NF-κB pathway leading to translocation of c-IAPs, and NF-κB-associated factors-2, -3 and -5 (TRAFs-2, -3 and -5) from the cytosol to the cell membrane and proteasomal or lysosomal-mediated degradation. In this manner, TRAFs are involved in multiple activation and inhibition signaling patterns [97], which regulate cellular recognition via their cytoplasmic interaction with other signaling molecules [98].

With respect to the role of TNFRI in apoptosis, the binding of TNF-α to the Type I TNF receptor (TNFRI) triggers phosphorylation (P) by IkB kinases of the inactive IκB/Rela/p50 NF-κB complex (Figure 1) with translocation of Rela/p65 and NFKB1/p50 to the nucleus. IκBα liberated from the inactive NF-κB complex undergoes proteasomal-mediated degradation. NF-κB-dependent apoptosis can be blocked by XIAP. As previously reported [99,100], XIAP was localized to both the cytoplasm and nucleus, but XIAP and another anti-apoptosis protein, survivin, were highly expressed in the cytoplasm of cells in active RA synovial tissue [101]. Second mitochondria-derived activator of caspase (caspase-8) inhibitor of apoptosis-binding protein with low pt (Smac/DIABLO) [38] mediated inhibition of XIAP activity may favor induction of apoptosis over cell survival under these conditions [31,38]. In addition, XIAP and other IAP proteins can directly inhibit caspase-3 and caspase-7 activity [102] as well as modulate the Bax/cytochrome C apoptosis pathway via XIAP-mediated inhibition of caspase-9 [103]. Thus, elevated levels of XIAP may be responsible for low caspase-3 activity and inhibition of apoptosis (i.e. "apoptosis-resistance") in RA synovium [31].

PI-3K/AKT/mTOR Pathway Activation

The involvement of the PI-3K/AKT/mTOR pathway (Figure 1) in promoting aggressive immune-cell and synoviocyte proliferation and survival, neoangiogenesis, apoptosis, and altered innate immunity in inflammatory arthritis was recently reviewed [64]. The salient features of this review can be summarized as follows in which both positive and negative responses by cells involved in inflammatory arthritis...
were noted: 1) PI-3K/AKT/mTOR activity was associated with an increase in neutrophil, macrophage and eosinophil chemotaxis, mast cell degranulation, as well as activation, maturation and survival of activated T- and B-cells; 2) activation of PI-3K/AKT/mTOR by Tumor Necrosis-Related Apoptosis-Inducing Ligand (TRAIL) and IL-15 resulted in exuberant RA-fibroblast-like synoviocyte proliferation and increased IL-17 production by CD4+ T-cells, respectively; 3) conversely, activation of PI-3K/AKT/mTOR could also cause suppression of immune-cell proliferation by dampening Forkhead box protein O (FoxO) transcription factor activity; 4) resistance of RA synoviocytes to Fas (CD95)-induced apoptosis (Figure 1) involving activation of extracellular S1P via PI-3K/AKT/mTOR-dependent-SphK1; 5) Involvement of PI-3K/AKT/mTOR in innate immune responses pertinent to inflammation was demonstrated by the capacity of

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**Figure 1:** An Apoptotic/Survival Cascade in Synovial Fibroblasts: Regulation via Fas (CD95), TNF-α and Growth Factor Receptors. Apoptosis or survival of synovial fibroblasts can be induced by several types of ligand-receptor interactions. These interactions may involve Fas, cytokine or growth factor receptor activation. P, site for MAPK-mediated phosphorylation.
activated PI-3K/AKT/mTOR to dampen TLR4-mediated immune cell activity and regulation of peptidoglycan-induced IL-6 production in human synoviocytes.

In other studies, Cejka et al. [104] showed that inhibition of mTOR activity through treating human TNF-transgenic arthritic mice with sirolimus or everolimus inhibited osteoclast development, bone erosions and cartilage loss. Of interest, fewer bone-derived osteoclasts were found ex vivo, which was traced to induction of osteoclast apoptosis in mTOR inhibitor-treated TNF-transgenic arthritic mice. Importantly, a correlation was found between activated mTOR signaling and the number of osteoclasts in RA patients. The results of this study were supported by a proposal made by Kim et al. [105] who have asserted that mTOR activity was crucial for osteoclast survival. Thus, Kim et al. [105] contended that in RA patients combination therapy with an mTOR inhibitor and vitamin D(3) could effectively block subchondral bone erosion. Lastly, a recently discovered cytokine, IL-22, was shown to significantly increase the proliferation of fibroblasts isolated from the skin of patients with psoriatic arthritis [106]. Moreover, proliferation was effectively blocked by NVP-BEZ235, a dual inhibitor of PI-3K/mTOR. The effect of NVP-BEZ235 was apparently dependent on AKT/mTOR activity since IL-22 induced AKT and mTOR phosphorylation in these fibroblasts and in normal human epidermal keratinocytes.

The PI-3K/AKT/mTOR pathway can also be activated by growth factors (Figure 1) such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) both of which play significant roles in RA [107-109] because they can induce immune-cell, synoviocyte or chondrocyte apoptosis and/or survival which may be regulated by activation of cellular AKT [107,108]. Thus, the growth factor pathway cascade is initiated when VEGF or FGF interacts with their respective receptor tyrosine kinases resulting in activation of PI-3Kγ, the intermediates phosphatidylinositol-phosphate-3 (PIP-3) and phosphoinosside-dependent protein kinase-1 (PDK-1) which then causes AKT1 activation [109]. Thus, when AKT1 is correctly oriented at the cell membrane through its capacity to bind to PIP-3, AKT1 is phosphorylated by its activating kinases, PDK-1 and mTOR complex 2 (mTORC2). In the context of RA, activated AKT1 phosphorolylates several downstream signaling proteins resulting in up-regulation of mTOR and down-regulation of Glycogen Synthase Kinase-3 (GSK-3), FKHR, also known as Forkhead box protein O1, and Bad [110,111], the latter a member of BH3-interacting death-domain antagonist protein family [30], as well as other signaling proteins [112]. For example, AKT1 can differentially regulate the transcription of anti-apoptosis (i.e. survival pathway) genes such as 14-3-3 [113] and androgen receptor (AR) also known as nuclear receptor subfamily 3, group C, member 4 [114] or pro-apoptosis-related genes such as tumor protein-73 (TP-73) [115] which is structurally related to the p53 tumor protein.

Conclusions

The JAK1/3 SMI, tofacitinib, was recently developed for the treatment of moderate-to-severe cases of RA in patients who have inadequately responded to conventional DMARDs or therapy with anti-rheumatic biological drugs. The approval of tofacitinib appears to have provided the impetus for future basic research aimed at discovering novel protein kinase-specific SMIs which may be targeted towards individual protein kinases or targeted as a dual protein kinase inhibitor such as the newly described N1-p-fluorobenzyl-cymserine which was reported to inhibit both p38 kinase and JNK as well as NF-xB [116].

In addition to the role played by pro-inflammatory cytokines in RA that cause deregulation of JAK/STAT [26-28] and SAPK/MAPK [117-121], the results of recent studies have focused on why the PI-3K/AKT/mTOR signaling pathway (Figure 1) may be the most suitable target in the development of SMIs for RA [104-106]. Certainly, additional targets for regulating abnormal JAK/STAT activity could also arise from a greater understanding of the negative regulators of JAK/STAT activation, including Suppressor of Cytokine Signaling (SOCS), protein phosphatases such as SHP-1, and Protein Activator of Activated STAT (PIAS) proteins which are also significantly deregulated in RA [26]. Thus, along with the aforementioned protein kinases, Syk, SphK1 and SphK2, TAK1, BMP and NIK which were also identified as participants in the RA process these enzymes could all become suitable targets for future medical intervention in RA.

However, enthusiasm for developing SMIs for any one specific protein kinase implicated in the pathophysiology of RA must be tempered and viewed with cautious optimism as a result of the recent evidence from the OSKIRA-I Phase III safety and tolerability study [122]. As previously discussed, Syk is a suitable target for RA intervention. This is because activation of Syk occurs in response to B-cell antigen receptor (BCR) engagement [42-46]. As such, activation of the BCR and Syk activation downstream of the BCR regulates B-cell development, and survival. In OSKIRA-I, fostamatinib (formally known as R788), an orally administered Syk SMI, improved the clinical symptoms of 923 RA patients who had not adequately responded to methotrexate. However, this clinical response was seen only at the American College of Rheumatology-20 (ACR20) criteria. Importantly, fostamatinib failed to improve the modified Total Sharp Score when compared to placebo at 24 wks at either of the two doses employed. The most common side-effects of fostamatinib in OSKIRA-I were hypertension, diarrhea, nausea, headache and nasopharyngitis. However, the results of previous clinical studies had also shown that fostamatinib was associated with increased rates of infection, elevated liver enzyme levels and neutropenia [56]. Therefore, additional data will have to be collected over a longer period of observation in order to determine the extent to which fostamatinib can improve clinical outcomes in RA at least at the ACR50 level. Whether or not a positive clinical response to fostamatinib will correlate with a reduction in subchondral bone erosions and/or inhibition of articular cartilage destruction must also be considered in judging the effectiveness of fostamatinib as an RA therapy.

Acknowledgements

The Arthritis Research Laboratory (ARL) was supported by an investigator-initiated project grant from Takeda Pharmaceuticals of North American and is presently supported by a research contract from Genentech/Roche Group to CJM. The ARL is also supported by the Visual Sciences Research Center Core Grant, P30-EY11373, from the National Eye Institute. We thank Mr. Evan C. Meszaros, M.S. for helpful discussions and for rendering the information in Figure 1. The author declares no conflict of interest.

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